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Bcl-2 Protein Expression and Long-Term Survival in Breast Cancer

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Bcl-2 gene product functions to prevent apoptosis in a variety of* in vitro *and* in vivo *experiments. The prognostic significance of Bcl-2 protein expression was investigated by immunocytochemistry from paraffin-embedded tissue in a series of 174 women with breast cancer, treated with radical surgery with or without regional radiotherapy, and who had been followed up for the median of 31 years or until death. A minority (25%) of cancers were entirely negative for Bcl-2 protein. Moderate to strong Bcl-2 protein expression (present in 46%) was strongly associated with several favorable prognostic features, such as a low mitotic count, high histological grade of differentiation, and lack of p53 protein expression (P < 0.0001 for each). It was also significantly associated with lack of tumor necrosis, a low S-phase fraction size, low cathepsin D expression, DNA diploidy, and the lobular histological type, but not with the primary tumor size or the axillary nodal status. Women with cancer with moderate to strong Bcl-2 protein expression had more favorable short-term (69% versus 46% alive at 5 years) but similar long-term (29% versus 33% alive at 30 years) disease-specific survival as those with cancer with weak or lacking expression. Bcl-2 protein expression did not have independent prognostic value in a multivariate survival analysis. We conclude that Bcl-2 protein is frequently expressed in breast cancer, and its expression is associated with favorable clinicopathological features. (Am J Pathol 1994, 145:1191–1198)

The *Bcl-2* proto-oncogene has been considered to be a cell death suppressor gene that regulates the programmed cell death called apoptosis.[1,2] All hematopoietic and lymphoid cells, many epithelial cells, and neurons contain *Bcl-2* oncoprotein.[3] The protein is found mainly in the periphery of mitochondria, on the perinuclear membrane, and in the endoplasmic reticulum.[4,5] About 70% of follicular lymphomas and 20% of diffuse B-cell lymphomas have high concentrations of *Bcl-2* protein as a consequence of the t(14;18)(q32;q21) translocation that positions the *Bcl-2* gene under the control of the strong immunoglobulin heavy-chain gene promoter.[6,7] The fused *Bcl-2*-Ig gene generates chimeric mRNAs that consist of *Bcl-2* at 5′ portion and immunoglobulin at 3′ portion, and the chimeric mRNA contains the *Bcl-2* coding frame for a 239-amino acid polypeptide. In consequence, high levels of *Bcl-2* protein are generated. However, a discrepancy in the relationship between the occurrence of t(14;18) translocation, *Bcl-2* gene rearrangement, and overexpression of *Bcl-2* protein has been found in lymphoma,[8,9] which suggests the existence of several molecular mechanisms for *Bcl-2* protein overexpression.

In low grade lymphoma, *Bcl-2* protein expression is thought to result in accumulation of cells by inhibition of apoptosis. Mice transgenic for the chimeric *Bcl-2*-Ig gene demonstrate extended B cell survival and follicular lymphoproliferation.[10] Epstein-Barr virus proteins increase the expression of *Bcl-2* in Burkitt's lymphoma cells, and protection from apoptosis is conferred through expression of the latent membrane protein LMP-1.[11,12] High concentrations of *Bcl-2* protect cells from apoptosis induced by c-*myc*.[13] However, different pathways for induction of apoptosis may exist, and only some of them may be affected by *Bcl-2* expression.[14]

The *Bcl-2* protein can be detected by immunocytochemistry using paraffin-embedded tissue sections as the starting material, which makes it possible to study the prognostic value of *Bcl-2* expression in dif-

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ferent types of human cancer using series with known outcome. In the present study we investigated the prognostic value of *Bcl-2* protein and its correlations with several clinicopathological variables in a series of patients with breast carcinoma who had a minimum of 26-year follow-up.

Materials and Methods

Patients

The present series was derived from a larger series (n = 439) encountered in the city of Turku, Finland, from 1945 to 1965. The series included 95% of all histologically diagnosed female breast carcinomas from this defined area and period and has been described in detail elsewhere.[15] Women with intraductal *in situ* cancer or Paget's disease of the breast (n = 15), bilateral cancer (n = 23), disseminated disease at the time of diagnosis (n = 31), or those who received only palliative treatment (n = 22) were excluded from the analysis. From the remaining 348 cases, one half (n = 174) selected at random were analyzed for *Bcl-2* expression.

In all cases the clinical records, autopsy protocols, and all histological sections were reviewed in order to find out the cause of death. Because the cause of death may sometimes be difficult to determine even if autopsy is done, we also calculated the relative survival rate by dividing the crude survival rate by the expected rate in the general female population, matched for age and the year of follow-up, which information was derived from the tables of the Central Statistical Office of Finland. The relative survival curve obtained by dividing the overall survival by the expected rate was found to follow closely the survival curve obtained by correcting for known intercurrent deaths, which excluded the presence of any major misclassification of causes of death in the series (data not shown).

The median follow-up period of the patients still alive was 31 years (range, from 26 to 43 years). Twenty-two patients (13%) were still alive, 111 (64%) had died from breast cancer, 7 (4%) from some cancer other than breast cancer, 33 (19%) from an intercurrent disease, and in 1 case the cause of death remained unknown. The median age at diagnosis was 54 years (range, from 28 to 89 yrs). Clinical staging was performed according to the postsurgical International Union Against Cancer Tumor-Node-Metastasis classification (1987). One hundred thirty-six women (78%) were treated with mastectomy and axillary evacuation, 30 (17%) had mastectomy only, and 8 (5%) had tumorectomy. Postoperative radiotherapy was given to 128 (74%) patients.

Histology

New hematoxylin-eosin stained slides were prepared from each tissue block, which were routinely fixed in neutral formalin and embedded in paraffin. The histological typing and grading of the tumors were performed with a slight modification of the World Health Organization classification.[16] Subsequently, the tumors were grouped into three types: 1) infiltrating ductal carcinoma not otherwise specified; 2) infiltrating lobular carcinoma; and 3) other special types (tubular, cribriform, medullary, papillary, and pure mucinous carcinomas).

Immunohistochemical Analyses of Bcl-*2 and p53*

4-μ sections were cut from paraffin blocks and stained with mAbs against *Bcl-2* and p53 oncoproteins. The sections were deparaffinized in xylene (2 × 15 minutes), transferred to 0.05 mol/L Tris-buffered saline (TBS) pH 7.40 to 7.60 through a graded ethanol series (3 minutes in each solution). After washing in TBS (3 × 5 minutes), the slides were heated in a microwave oven at 650W (2 × 5 minutes) in 10mM sodium citrate buffer at pH 6.0 and allowed to stay at room temperature for 20 minutes. After washing in TBS (3X) and an ascending alcohol series the endogenous peroxidase was blocked with 0.3% H_2O_2 for 30 minutes. The samples were transferred to TBS through a descending alcohol series and stained with the avidin-biotin complex method. After blocking unspecific staining by incubating the slides with normal horse serum (Vector Laboratories Inc., Burlingame, CA), the two separate slides were incubated in BSA overnight at 4 C with the primary mouse MAbs for p53 (clone Do-7, DAKO, Copenhagen, Denmark) diluted in 1:800, or *Bcl-2* (clone 124, DAKO) diluted in 1:80. Horse biotinylated antimouse IgG in 2% BSA was applied onto the sections at room temperature for 30 minutes, and thereafter the slides were incubated with the streptavidin-peroxidase conjugate (Vector Laboratories) for 30 minutes at room temperature, and stained with 3,3′-diaminobenzidine (Polyscience Inc., Warrington, PA) as the chromogen for 5 minutes. All incubations were carried out in a humidified chamber. Finally, a slight Mayer hematoxylin counterstain was applied, followed by dehydration and mounting of the sections. A section treated with 2% BSA in TBS without the primary antibodies served as a negative

control. Sections from human follicular lymphoma served as a positive control for *Bcl*-2 protein staining, and sections from human ovarian carcinoma known to be positive for p53 oncoprotein served as positive controls for p53 protein staining. In addition, sections from normal breast tissue were stained with mAbs against *Bcl*-2 and p53 oncoproteins.

All slides were evaluated for immunostaining in a blinded fashion without any knowledge of the clinical outcome or other clinicopathological data. Staining both for *Bcl*-2 and p53 were visually classified into four groups: no staining present in any of the breast cancer cells (–), slight staining in some cells or in most of the cells (+), moderately strong staining (++), or strong staining present in almost all cells (+++). Classification was done by a senior pathologist with special interest in breast cancer histology (ST). To test the repeatability of classification, another investigator (LP) classified the same slides without knowledge of the former classification or other data. The great majority of slides stained for *Bcl*-2 oncoprotein (91.1%) were similarly classified by both investigators either as negative to slightly positive (– or +) or to moderately to strongly positive (++ or +++; for grouping, see below). The classification of the senior pathologist was used in statistical analyses.

DNA Flow Cytometry

DNA flow cytometry was carried out as described in detail previously[17] from dewaxed, rehydrated and pepsin-treated 50-μ sections of paraffin-embedded tissue. DNA was stained with propidium iodide. The size of the S-phase fraction was calculated by the rectangular method. The mean coefficient of variation of the diploid peaks was 7.6% (SD, 2.0%). DNA ploidy was not determined in 22 cases and S-phase fraction was not determined in 92 cases because of lack of tissue, overlapping stemlines, presence of excessive background debris, or uncertainty of histogram classification.

Statistical Analyses

Statistical analyses were done with the BMDP computer program (BMDP Statistical Software, Department of Biomathematics, University of California, Los Angeles, CA). Frequency tables were analyzed with the χ^2 test. Cumulative survival was estimated with the product-limit method, and comparison of cumulative survival between groups was performed with the log-rank test. Survival corrected for intercurrent deaths was used in statistical calculations, and women who died from causes other than breast cancer were withdrawn from the analysis at the date of death. Women who died with breast cancer with distant metastases based on clinical or autopsy evidence were considered to have died from breast cancer. The relative importance of prognostic factors was analyzed using Cox's proportional hazard model (BMDP 2L). All *P* values are two-sided.

Results

Expression of Bcl-*2 Protein in Breast Cancer*

Forty-three (25%) cancers showed no immunoreactivity for Bcl-2 protein in malignant epithelial cells (–), and 51 (29%) had slight (+), 51 (29%) moderate (++), and 29 (17%) strong staining (+++) for *Bcl*-2 protein. Immunostaining resulted in cytoplasmic immunoreactivity. The non-neoplastic lobular and ductal epithelial cells and mature lymphocytes expressed the *Bcl*-2 protein usually with a strong staining intensity (Figure 1).

Clinicopathological Features of Tumors with High Bcl-*2 Expression*

Moderate to strong expression of *Bcl*-2 protein (++; +++) was more common in the lobular histological type (80%) than in the ductal type (37%, $P = 0.0002$), and it was more common in women older than the median (54 years) than in those younger than the median (55% *versus* 36%, $P = 0.01$). *Bcl*-2 protein expression was strongly associated with several favorable prognostic features, such as a low cell proliferation rate estimated either by the mitotic count ($P < 0.0001$) or the S-phase fraction size ($P = 0.001$), high histological grade of differentiation ($P < 0.0001$), and lack of p53 protein expression ($P < 0.0001$). It was also significantly associated with lack of tumor necrosis, low stromal cell cathepsin D expression, and DNA diploidy, but not with the primary tumor size or the axillary nodal status (Table 1).

Survival

The presence of cytoplasmic *Bcl*-2 expression was associated with favorable outcome. Cancers with no or weak staining (– or +) had similar outcome, and cancers with stronger staining (++ or +++) also had similar prognosis. Therefore, these groups were combined in survival analyses. Women with cancer with strong *Bcl*-2 protein expression had a 69% 5-year and 29% 30-year survival rate corrected for intercurrent

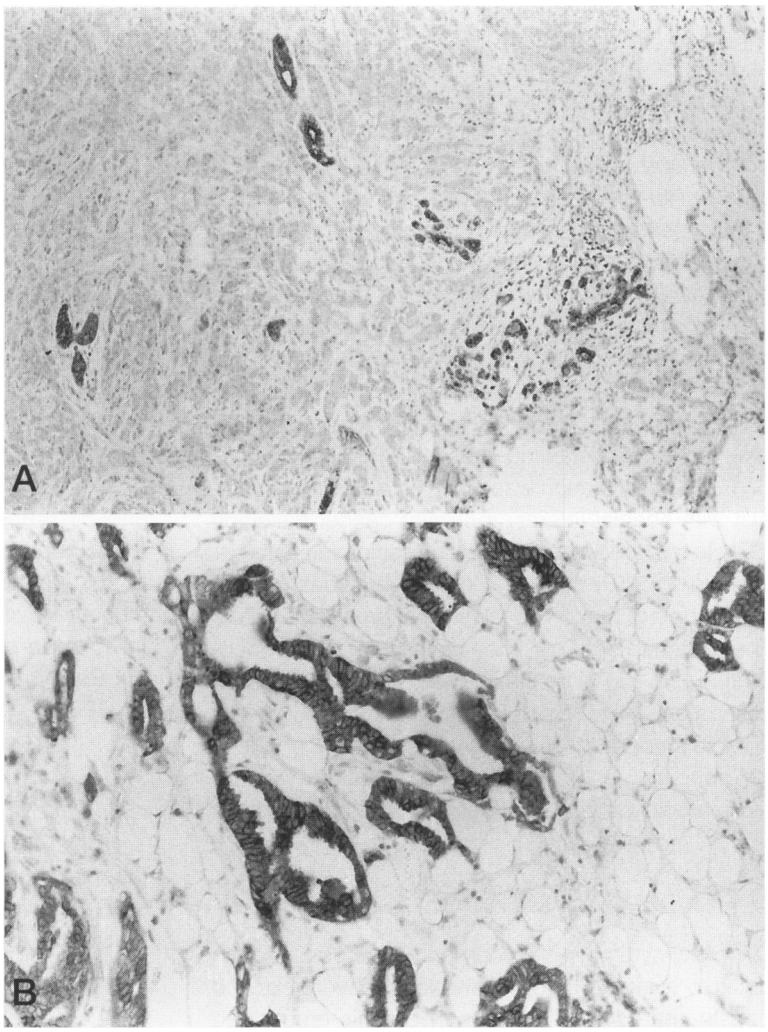

Figure 1. Top: *Breast carcinoma cells with a negative staining result for* Bcl-*2. Note the positive reaction in non-neoplastic lobular and ductal epithelial cells.* Bottom: *Tubule-forming breast carcinoma cells with a strong positive cytoplasmic staining result. Hematoxylin counterstain; original magnification* ×*100.*

deaths, whereas those with cancer with weak or lacking *Bcl-2* expression had a 46% 5-year and 33% 30-year survival rate ($P = 0.006$). A similar association with survival was found if overall survival was analyzed instead of survival corrected for intercurrent deaths (Figure 2).

Moderate to strong staining for *Bcl-2* was associated with favorable outcome among axillary node-positive patients (pN+, n = 89, $P = 0.0002$), but no significant association between *Bcl-2* staining and survival was found among the node-negative cases (pNO, n = 47, $P = 0.27$). Moderate to strong *Bcl-2* expression was associated with favorable survival also in the subgroup of women with the ductal histological type (n = 135, $P = 0.02$).

To assess the independent prognostic value of *Bcl-2* protein expression it was entered with other prognostic factors in a multivariate analysis. Several classical prognostic factors were associated with unfavorable survival in the present series, and they included the presence of axillary nodal metastases at diagnosis (pN+ *versus* pNO), a large primary tumor size (pT3-4 *versus* pT2-1), low histological grade of differentiation (Grade III *versus* Grade II *versus* Grade I), a high mitotic count (more than three mitoses per high power field *versus* two to three *versus* rare), presence of tumor necrosis (necrosis *versus* no necrosis), and the ductal histological type (ductal *versus* lobular *versus* the specialized types, P was < 0.0001 for each of these factors in a univariate survival analysis). When *Bcl-2* expression was tested together with these factors using the Cox's stepwise analysis, axillary nodal status (relative risk, 3.9; 95% confidence interval, 2.2 to 6.8), the primary tumor size (2.4; 1.6 to 3.7), tumor necrosis (2.3; 1.5 to 3.6), histological type (1.8; 1.1 to 2.9), and mitotic count (1.3; 1.0 to 1.7) had independent prognostic value, whereas *Bcl-2* protein expression did not. If overall survival was assessed instead of survival corrected for intercurrent deaths, the same five factors remained as the only indepen-

Table 1. *Correlation of Bcl-2 Protein Expression with 11 Clinicopathological Features in Breast Cancer*

Feature	*Bcl-2* Protein Expression: Negative N (%) −/+	*Bcl-2* Protein Expression: Positive N (%) ++/+++	*p*
Mitotic count/high power field (n = 174)			
Rare	9 (17)	44 (83)	
2–3	41 (64)	23 (36)	
>3	44 (77)	13 (23)	<0.0001
Histological grade (n = 174)			
Grade I	6 (17)	30 (83)	
Grade II	32 (46)	37 (54)	
Grade III	56 (81)	13 (19)	<0.0001
p53 protein expression (n = 161)			
No	37 (41)	54 (59)	
Slight	16 (48)	17 (52)	
Strong	32 (86)	5 (14)	<0.0001
Tumor necrosis (n = 174)			
None	37 (41)	53 (59)	
Spotty/moderate/severe	57 (68)	27 (32)	0.0004
Histological type (n = 174)			
Ductal	82 (61)	53 (39)	
Lobular	5 (20)	20 (80)	
Special	7 (50)	7 (50)*	0.0008
DNA ploidy (n = 157)			
Diploid	18 (37)	31 (63)	
Nondiploid	67 (62)	41 (38)	0.003
S-phase fraction (n = 101)			
≤9% (median)	17 (34)	33 (66)	
>9%	34 (67)	17 (33)	0.001
Stromal cell cathepsin D (n = 163)			
Negative staining	12 (36)	21 (64)	
Positive staining	76 (58)	54 (42)	0.02
Age at diagnosis (n = 174)			
≤54 (median)	53 (64)	30 (36)	
>54	41 (45)	50 (55)	0.01
Axillary nodal status (n = 136)			
pN0	24 (51)	23 (49)	
pN+	53 (60)	36 (40)	0.34
Primary tumor size (n = 173)			
pT1	10 (50)	10 (50)	
pT2	52 (51)	50 (49)	
pT3	23 (64)	13 (36)	
pT4	9 (60)	6 (40)	0.55

* Two out of the four tubular carcinomas and all five medullary carcinomas had negative (−/+) *Bcl-2* protein expression.

dent factors. Similarly, *Bcl-2* protein expression did not have independent prognostic value when tested among patients with node-negative disease or among those with node-positive disease.

Discussion

In the present series *Bcl-2* protein was frequently expressed in the cytoplasm of breast cancer cells. *Bcl-2* expression was particularly common in well differentiated carcinomas (83% moderately or strongly positive). The cause of abnormal *Bcl-2* oncoprotein expression in breast cancer is not known, but *Bcl-2* oncoprotein may be expressed without the 14;18 translocation,[18] and post-transcriptional regulation mechanisms for controlling of *Bcl-2* oncoprotein expression may exist. The relative absence of *Bcl-2* protein found in poorly differentiated breast cancers as compared with the well differentiated ones suggests down-regulation of the protein with tumor progression. This, in turn, could lead into induction of apoptosis and shortened survival of cancer cells. However, another type of cell death, tumor necrosis, is also more common in poorly differentiated breast cancer than in well differentiated cancer, and both ways of cell death may be counteracted by the high cellular proliferation rate seen in poorly differentiated cancer and in *Bcl-2* protein-negative breast cancer (Table 1). In line with these findings, the number of apoptotic bodies has been found to be high in lymphomas with a high proliferation rate.[19]

p53 may not only function as an inhibitor of cell division, but in other situations may also act as a gene that causes apoptosis,[3] and overproduction of normal p53 protein in a myeloid cell line induces rapid cell death by apoptosis.[20] In line with our findings, an inverse relationship between abnormal p53 protein expression and *Bcl-2* protein expression has recently

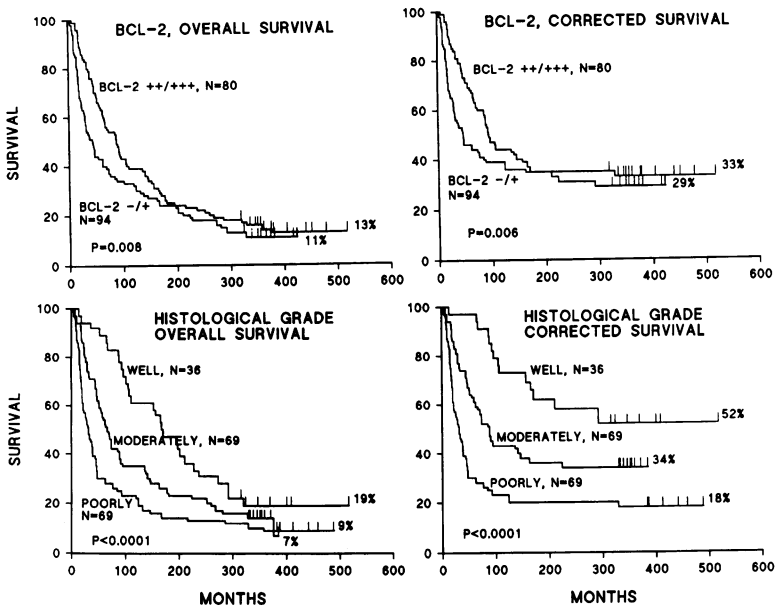

Figure 2. *Influence of* Bcl-*2 protein expression on survival among 174 women with breast cancer. The patients still alive are indicated by a bar. The influence of histological grading on survival is shown for comparison.*

been found by others in non-Hodgkin's lymphoma[21] and in breast cancer.[22,23] In breast cancer, *Bcl-2* expression is associated with estrogen receptor positivity.[22,23]

Bcl-2 was more often expressed in the lobular than in the ductal histological type. The reasons for this remain unexplored and may reflect the different origin of the two histological types of breast cancer. However, lobular carcinoma showed better grade of differentiation (assessed from the nuclear grade) than the ductal type in the present series (60% *versus* 10% were well differentiated, $P < 0.0001$). Hence, the more frequent expression of *Bcl-2* protein in lobular cancer in the present series may be associated with the better grade of differentiation and less pronounced down-regulation of *Bcl-2* gene expression in this histological subtype.

Although *Bcl-2* protein expression was associated with adverse outcome in a univariate analysis, it did not turn out to be an independent prognostic variable in a multivariate analysis when compared with six more traditional prognostic factors. Similarly, Silvestrini et al[23] found *Bcl-2* protein expression to be associated with favorable survival in a univariate analysis, but it was not an independent prognostic factor in a multivariate analysis. Hence, *Bcl-2* expression may not find clinical use as a prognostic factor in breast cancer. Lack of *Bcl-2* expression may be an adverse prognostic factor also in non-Hodgkin's lymphoma,[19] although in follicular lymphoma the prognostic value of *Bcl-2* expression has been found to be small.[24] *Bcl-2* protein has recently been investigated in non-small cell lung carcinoma, where survival at five years was found to be higher among patients with *Bcl-2*-positive tumors.[25] Although the clinical significance of *Bcl-2* expression may depend on the tumor type, our present findings in breast cancer suggest that the survival advantage associated with *Bcl-2* expression may disappear after the first 10 years of follow-up (Figure 2), and long-term follow-up may be needed to assess fully the prognostic significance of *Bcl-2* protein expression. Expression of *Bcl-2* may, however, be of greater interest as a predictive factor for response to hormonal or chemotherapy, because many drugs induce apoptosis. For example, transfection of the *Bcl-2* gene into a human pre-B-cell leukemia line resulted in a higher frequency of cell regrowth after exposure to several cytotoxic drugs than was observed in the control cultures.[26]

Although we obtained good correlation in assessment of *Bcl-2* protein expression between two independent classifiers, the visual classification system is somewhat subjective. We tested another method of classification, where cancers with ≤10% positive carcinoma cells for *Bcl-2* protein were considered as negative (n = 64) and those with >10% of positive cells as positive (n = 110). It turned out that patients with >10% of positive cancer cells had more favorable survival than those with ≤10% of positive cells in a univariate analysis with a *P* value of 0.007, a value similar to the value (0.006) we obtained with the classification system described in *Materials and Methods*. It may also be argued that in some cases the negative staining result for *Bcl-2* protein may have been caused by lack of tissue immunoreactivity. However, 25 of our 43 entirely negative cases for *Bcl-2*

stained positively for p53, 14 of the remaining 18 cases had positively stained tumor infiltrating lymphocytes, and 2 other cases were positive in immunostaining for vimentin and cytokeratin. Hence, lack of tissue immunoreactivity does not explain negative immunostaining for *Bcl-2* protein.

In conclusion, *Bcl-2* protein is often expressed in breast cancer, and its expression is associated with several favorable prognostic factors, such as high histological grade of differentiation, low cell proliferation rate, and the lack of tumor necrosis. *Bcl-2* protein expression is inversely associated with expression of the mutated p53 expression, which, in turn, is associated with increased cell proliferation rate and generally poor outcome.[27] Breast carcinomas that express *Bcl-2* protein strongly or moderately strongly have more favorable outcome than those that express it weakly or not at all, but the long-term prognosis of breast cancer does not appear to be influenced by *Bcl-2* oncoprotein expression.

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